DIPHTHERIA TOXOID AS AN IMMUNISING AGENT.

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IN a previous paper (Glenny, Allen and Hopkins, 1923) methods have been described for testing diphtheria toxin-antitoxin mixtures for use in active immunisation against diphtheria. By means of the methods there described it is now possible to determine the relative antigenic values of mixtures of toxin and antitoxin of varying strength and constitution, and of modifications of toxin. An ideal immunising agent is one that presents the highest antigenic strength but does not cause any injurious effects. The object of this present paper is to show what work has been done towards producing an ideal immunising agent against diphtheria.

Most of our knowledge of the relative immunising values of different toxins has been obtained from the routine immunisation of horses for the large scale production of diphtheria antitoxin. Experience has shown that the immunising value of a batch of toxin depends upon its specific antigenic value in relation to its toxicity. In this connection, by toxicity is meant not only specific toxicity, *i. e.* the number of minimal lethal doses of diphtheria toxin present, but also non-specific toxicity due mainly to broth constituents. When we are dealing with toxin-antitoxin mixtures for human use it is necessary also to consider the amounts of horse-serum present when given to serum sensitive subjects, and also of the "pseudo" constituent in the toxin when the mixture is used to immunise a "combined" (pseudo and positive) Schick-reactor. The specific antigenic value of a toxin or of a toxin-antitoxin mixture depends upon the number of free binding units, whether of toxin or toxoid. For the purposes of this paper the term "toxoid" will be used in its general sense for any modification of toxin that will still combine with antitoxin but is no longer fatal to guinea-pigs. That toxoid is of equal specific antigenic value to toxin has long been known; in 1904 one of us produced diphtheria antitoxin containing over 1000 units per c.c. from a horse injected only with diphtheria toxin so modified that the M.L.D. for a guinea-pig of 250 gm. weight was 0·3 c.c. and the L 0 dose 0·5 c.c.; a few years later similar high value antitoxin was obtained from a horse injected with modified toxin, of which 5·0 c.c. would not kill a guinea-pig. The immunising value of modified toxin rich in toxoid is greater than that of toxin because, while the specific antigenic value remains the same, the specific toxicity is reduced or absent. When we are dealing, however, with hyper-immunisation, specific toxicity is of less importance, owing to the presence in the animal of sufficient circulating antitoxin to neutralise most of the specific toxin. Yet it is

interesting to note in passing that a small local injection is not all neutralised by circulating antitoxin, although the total antitoxin present in the animal may be enough to neutralise many thousand times the amount of toxin injected. The Schick reaction in man is a typical example of this; a man with five litres of blood containing $\frac{1}{100}$ unit of antitoxin per c.c. gives a Schick-positive reaction when injected with that amount of toxin which would be completely neutralised by $\frac{1}{1000}$ unit of antitoxin, *i.e.* $\frac{1}{500000}$ of the total circulating antitoxin in the patient. Again, immunised horses containing, say, 30 litres of blood have developed diphtheria paralysis after an injection of toxin that could be completely neutralised by 1 c.c. of their blood.

The use of modified toxin in toxin-antitoxin mixtures for human use enables a far greater number of binding units, *i. e.* a greater specific antigenic strength, to be presented without any increase in specific toxicity. It may be possible shortly to use toxin so modified that it will be completely non-toxic without the addition of antitoxin. This would constitute a marked advance in the prevention of diphtheria; the presence of horse-serum in a toxin-antitoxin mixture might possibly cause reactions in serum-sensitive subjects, and possibly sensitise others not already sensitive, although no worker has as yet found evidence of sensitisation by such small doses of serum in a human subject. The use of modified toxin, however, reduces the amount of serum necessary in a mixture, because such modified toxin need not be fully neutralised; only sufficient antitoxin need be added to reduce the residual toxicity below a certain level.

The cause of the variation in non-specific toxicity of different batches of toxin is not known, nor is there any convenient test beyond that of large scale immunisation of horses. In our experiences two batches of toxin may have identical values for M.L.D., L 0 and L +, and so presumably have the same specific antigenic values, and yet differ so markedly in their true immunising property that one may be regarded as almost worthless and the other as extremely good. It is not yet known whether non-specific toxicity is due entirely to absolutely non-specific broth constituents or whether bacterial protein may play some part. In toxin-antitoxin mixtures intended for human use dilutions of the mixtures must lessen the non-specific toxicity.

The original American standard for specific toxicity of a toxin-antitoxin mixture was that 1 c.c. should cause no ill-effects in a guinea-pig, while 5 c.c. should kill in 10 days or more. Later the specific antigenic value was increased by using mixtures with more free toxin, and it was found that mixtures were safe for human use if 1 c.c. did not kill guinea-pigs in less than 10 days. A still greater improvement was made by using dilutions of more toxic mixtures. We, however, have endeavoured to increase the number of binding units in mixtures without making them any more toxic, and have continued to accept the early American standard of toxicity.

The modification of toxin used in the experiments recorded in this paper was prepared by adding 0·1 per cent. formalin to diphtheria toxin and exposing it for 4 weeks at a temperature of 37° C. before removing to the cold room. The original values of the toxin showed an M.L.D. of 0·0025 c.c.; L 0, 0·15 c.c.; and L+, 0·2 c.c. After treatment the M.L.D. was 0·2 c.c.; the L 0, 0·3 c.c.; and the L+, 1·0 c.c.

The routine method now adopted by us for testing antigenic values has already been described by us (Glenny, Allen and Hopkins, 1923). This method consists in injecting guinea-pigs with a single dose of the toxin-antitoxin mixtures or toxin under test, resting the animals for 3 weeks, and then injecting at weekly intervals with Schick doses of toxin until the animals fail to show a reaction. The number of Schick doses so given, until the animal is sufficiently immune to show no reaction, is an index of the antigenic value of the material under test, and is now termed by us the "immunity index." Thus, if a guinea-pig was injected with 1·0 c.c. of a certain toxin-antitoxin mixture, and 3 weeks later the first Schick injection gave a positive reaction, and the following week the second Schick injection showed no reaction, then the immunity index for 1·0 c.c. of that mixture would be recorded as 2.

Occasionally in experimental work we have endeavoured to determine whether a substance has any value as an antigen to diphtheria. If the antigenic value is very low many Schick injections will be needed before the immunity of the test-animal is raised to the Schick-negative level; if the substance under test has no antigenic value, the immunity index will be the same number of Schick doses of diphtheria toxin that would need to be given at weekly intervals to a normal guinea-pig in order to raise its immunity to the Schick-negative level. It is necessary, therefore, to know the effect of weekly injections of Schick doses of toxin upon normal guinea-pigs.

In all experimental work on animals we have adopted as the Schick dose of toxin the amount that is just neutralised by $\frac{1}{1000}$ unit of antitoxin. This level was adopted because it represented the binding-unit content of $\frac{1}{50}$ M.L.D. of a well-matured toxin used in our first experiments, and, as we have already pointed out (Glenny and Allen, 1922), the level of immunity detected by a Schick test depends upon the number of binding units injected and not upon the toxin content alone. It was found that the number of weekly Schick doses necessary to immunise a guinea-pig varied according to the toxin used. Table I shows that a relatively fresh toxin, of which $\frac{1}{30}$ M.L.D. was equivalent to $\frac{1}{1000}$ unit of antitoxin, gave an average index of 31, while the shortest time in which a guinea-pig injected weekly with this dose became immune was 20 weeks. An old matured toxin, containing $\frac{1}{50}$ M.L.D. to the Schick dose, gave an average index of 12, while the lowest record for the toxin was 9. With the modified toxin one guinea-pig, when given only $\frac{1}{200}$ M.L.D. each week, was sufficiently immune to give no reaction to the sixth weekly injection, while the majority were immune by the time they received the seventh injection.

TABLE I.

Toxin number.	Description.	Volume injected.	Fraction of M.L.D.	Average index.	Lowest index.
J. 3284 .	Fresh toxin	0·0001 c.c.	$\frac{1}{30}$	31	20
J. 1915 .	Old toxin	0·0004 c.c.	$\frac{1}{50}$	12	9
Y.M.B. 101	Modified toxin	0·001 c.c.	$\frac{1}{200}$	7	6

If so few injections of so small an amount of "toxoid" will immunise a guinea-pig, we may hope in the course of time so to improve the methods of

diphtheria prevention that a single dose of modified toxin will act both as a Schick test and as an immunising agent.

The antigenic values of one or two injections of modified toxin acting as a primary stimulus were measured by the method already described, and the results are recorded in Table II.

TABLE II.

Dose.	No. of experiments showing an immunity index of—						
	1	2	3	4	5	Over 5.	Total.
Two injections of 0·01 c.c. .	—	4	2	—	—	1	7
One injection of 0·05 ,, .	—	15	9	5	5	5	39
,, ,, 0·1 ,, .	2	—	—	—	—	—	2
,, ,, 0·2 ,, .	1	1	—	—	—	—	2

The general condition of the test animals has a great influence upon the apparent immunity index of an antigen. A good antigen may occasionally show a poor index; thus in Table II two injections of 0·01 c.c. modified toxin have yielded a bad index once in seven experiments. A large number of tests have been made at different times upon the immunity index for a single dose of 0·05 c.c. because we have found that this is a useful critical dose, sufficiently effective as a stimulus to yield a very good index of 2 on many occasions, while at the same time the dose is not excessive. The minimal dose that will constantly yield a good index under good conditions will frequently yield a poor index if the test animals are subject to adverse conditions; the immunity index of a larger dose is not so easily affected. For this reason we use a dose of 0·05 c.c. of this modified toxin when testing the conditions affecting the production of immunity. The experiments quoted in the table refer only to those guinea-pigs that were not injected with other substances to affect the rate of production of immunity, but include all control animals and those of varying weight. Eight out of the ten experiments showing an immunity index of 4 or 5 occurred in a single group of animals injected at a time when feeding conditions were not good, and, consequently rate of growth was slow and infection rate high. The antigenic value of a single dose containing 0·05 c.c. of modified toxin is at least as good as that of the majority of batches of toxin-antitoxin mixtures which we have tested. Such a dose of unneutralised modified toxin is not more specifically toxic than a toxin-antitoxic mixture, contains from one-tenth to one-twentieth of the non-specific constituents, and in addition contains no horse-serum. Four guinea-pigs were injected with higher doses, and it will be seen from Table II that three out of four guinea-pigs injected with from ½ to 1 M.L.D. were Schick negative when first tested 3 weeks later, while the fourth animal was negative at the second test made 4 weeks after the primary stimulus.

The action of toxoid as a primary stimulus has already been described by one of us (Glenny and Sudmersen, 1921), when it was pointed out that "three weeks after a single dose of from 0·5 c.c. to 2·0 c.c. of an old formalinised toxin (L 0 dose about 1·0 c.c.; M.L.D., 5·0 c.c.), a number of guinea-pigs survived 2 M.L.D. of toxin without any local reaction." One guinea-pig that

had received four injections of toxoid over a long period of time reached an antitoxic titre of 8·5 units per c.c.

In the preparation of toxin-antitoxin mixtures from unmodified toxin there is very little margin between mixtures that are too toxic and mixtures so over-neutralised that they are of very low antigenic value. With modified toxin the differential region between L 0 and L + is so great that there is a very large margin of safety in the preparation of mixtures.

TABLE III.

Dose injected: 0·1 c.c. of a mixture containing—	Number of experiments showing an immunity index of—						
	1	2	3	4	5	Over 5.	Total.
0·1 c.c. antitoxin (27 units) + 10·0 c.c. modified toxin (approx. L 0)	—	2	4	—	—	—	6
0·1 „ „ 14·0 „ „	—	2	—	—	—	—	2
0·1 „ „ 20·0 „ „	—	3	1	—	—	—	4
0·1 „ „ 25·0 „ „	—	1	2	—	—	—	3
0·1 „ „ 30·0 „ „ (approx. L+)	1	6	—	—	—	—	7
0·1 „ „ 42·0 „ „	—	2	—	1	—	—	3
0·1 „ „ 50·0 „ „	—	—	2	—	—	—	2

Table III shows that mixtures may be prepared containing from 10 to 50 c.c. of modified toxin to the same amount (0·1 c.c.) of a certain antitoxin, and with all mixtures throughout this range the immunity index for 0·1 c.c. of the mixture is as good as that for 1·0 c.c. of the majority of the mixtures prepared from unmodified toxin. Several of the mixtures were tested at lower doses; thus the mixture containing 42 c.c. of modified toxin and 0·1 c.c. of antitoxin was injected in doses of 0·01 c.c. into 3 guinea-pigs; 2 gave an immunity index of 2 and 1 of 4. In this mixture the toxoid was only neutralised to the extent of about 20 per cent. The specific toxicity of the mixture was such that when diluted 1 in 10 the mixture would correspond with the U.S.A. standard for toxin-antitoxin mixtures, yet a high index is given when diluted 1 in 100.

The effect of modified toxin as a secondary stimulus to actively immune rabbits is shown by Table IV.

TABLE IV.

Rabbit.	Antitoxic value before injection in units per c.c.	Volume of modified toxin injected.	Maximum value after injection in units per c.c.	Time interval.
10	0·08	5·0 c.c.	12·0	8 days.
42	0·0005	0·05 c.c.	2·75	6 „
55	0·0005	0·05 c.c.	3·75	6 „
99	0·015	0·1 c.c.	1·75	11 „
120	0·0005	0·05 c.c.	0·04	8 „
121	0·0005	0·05 c.c.	0·03	6 „
148	0·0005	0·05 c.c.	9·0	10 „
151	0·005	0·05 c.c.	4·5	8 „

Further work is being carried out along the following lines:

(1) Purification of toxin by methods of concentration which increase the number of binding units per milligramme of nitrogen about 40 fold.

(2) Using toxin so further modified that it is not toxic even in large doses.

(3) Investigation of the spectrum of partial neutralisation in relation to antigenic values.

Our thanks are due to our medical colleagues Drs. O'Brien, Eagleton and Okell for the information that a mixture of modified toxin and antitoxin (that containing 20·0 c.c. of "toxoid" to 0·1 c.c. of antitoxin and diluted 1 in 10) has given exceptionally promising results when employed for human immunisation.

SUMMARY.

The value of a diphtheria toxin for immunising purposes increases as toxin changes into toxoid (or toxone).

Toxins can be changed into toxoid by the action of formalin.

Such modified toxin containing 1·5 M.L.D. per L 0 and 5 M.L.D. per L+ has been used for the production of toxin-antitoxin mixtures showing a high immunity index.

It is hoped by further reduction in toxicity and by concentration to produce an immunising agent of far greater efficiency than any yet employed for protection against diphtheria.

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